ON THE EVOLUTION OF HEMOGLOBIN. RESPIRATORY PROPERTIES OF THE HEMOGLOBIN OF THE CALIFORNIA HAGFISH, POLISTOTREMA STOUTI[1]

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The Cyclostomata, which is composed of the hagfishes (Myxinoidia) and the lampreys (Petromyzontia), is considered on morphological (Young, 1950) and biochemical (Florkin, 1949; Wald, 1952) evidence to be the most primitive group of living craniate vertebrates. Whereas the molecular weight of vascular hemoglobin of all known non-cyclostome vertebrates corresponds to four oxygen-combining units (hemes) per molecule, the hemoglobin of both hagfishes and lampreys consists of but a single heme per molecule (Svedberg, 1933; Lenhert, Lowe and Carlson, 1956). With regard to amino acid composition, cyclostome hemoglobin appears to be intermediate between vertebrate and invertebrate hemoglobins (Florkin, 1949).

The oxygen equilibrium of hemoglobin solutions prepared from the blood of the sea lamprey, *Petromyzon marinus*, has been recently studied (Wald and Riggs, 1951). This hemoglobin possesses a hyperbolic oxygen dissociation curve (as would be expected on the basis of the above-mentioned molecular weight), a low oxygen affinity, and an extremely large Bohr effect. Wald (1952) has claimed that the evolution of hemoglobin has proceeded in three stages (p. 366): "(1) the heme enzymes of cellular respiration [cytochrome oxidase being considered as the phylogenetic precursor of hemoglobin (Wald and Allen, 1957)]; (2) cell and tissue hemoglobins concerned primarily with oxygen storage; and, (3) circulatory hemoglobins, concerned with the transport of oxygen from the lungs, gills, and skin to the internal tissues." Wald emphasizes that in this progression the three main biochemical aspects of the combination of hemoglobin with oxygen are altered: (1) the oxygen dissociation curve changes from hyperbolic to sigmoid—*i.e.*, heme-heme interaction develops; (2) the affinity for oxygen decreases—*i.e.*, the oxygen molecule is held less tightly to the heme; and (3) the oxygen affinity becomes a function of pH—*i.e.*, a Bohr effect is developed.

In view of these facts concerning the cyclostomes, and Wald's (1952) theory on the evolution of hemoglobin, it is of interest to evaluate the oxygen equilibrium of the hemoglobin of the California hagfish, which is perhaps an even more primitive vertebrate than the lamprey.

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Materials and Methods

Blood was obtained from 30 specimens of the California hagfish, *Polistotrema stouti* (Lockington), formerly called *Bdellostoma* or *Eptatretus stouti*. The blood was collected by placing capillary tubes adjacent to the severed ends of blood vessels; no anticoagulant was necessary, for the blood of this animal has little clotting ability. The blood was occasionally contaminated with a trace of the ubiquitous slime; this was easily removed by diluting the blood with isotonic phosphate-buffered saline and filtering through glass wool. In several cases blood from a single animal provided enough hemoglobin for a single oxygen equilibrium determination. However, the hemoglobin concentration is low (3–4%), and usually less than 1 cc. of blood is available from each animal; therefore, blood from several animals was often pooled. Erythrocytes were either (1) washed once in 15 cc. of isotonic saline and used immediately for determination of the oxygen equilibrium of dilute erythrocyte suspensions (equivalent to whole blood), or (2) washed two more times and then hemolyzed. Distilled water hemolysis did not give satisfactory results; up to 80% of the hemoglobin remained inside the cell. Therefore, a trace of powdered saponin was added to a suspension of one volume of cells to two volumes of distilled water. Several hours later the hemoglobin solution was separated as a supernatant by centrifugation, diluted with an equal volume of potassium phosphate buffer ($\Gamma/2 = 0.4$) of the desired pH, and then filtered through Whatman No. 5 paper. Such a hemoglobin solution is stable for days, although (except where specifically indicated) it was used immediately for oxygen equilibrium measurements. Preparation of hemoglobin was at 0–1° C., except for centrifugation at 8–12° C.

Oxygen equilibria were evaluated as in previous studies (Manwell, 1958a, 1958b). Erythrocytes were suspended in 9 parts isotonic sodium chloride (0.54 *M*) to 1 part potassium phosphate buffer of desired pH. To eliminate rapid settling of cells during spectrophotometric determination of oxyhemoglobin, and to reduce light-scattering effects, many erythrocyte suspensions were diluted 3 : 1 with Karo (a mixture of sugars, dextrins, and soluble starch, which has a high refractive index and thus effects a partial clarification of the cell suspension). Erythrocytes could be stored for a week in such a medium without hemolysis, although this undesirable effect took place to a slight extent in a few experiments involving prolonged equilibration. Therefore, some experiments were performed on cells simply suspended in buffered saline to which a trace of powdered bovine serum albumin was added to increase cell stability; in these instances absolutely no hemolysis was observed during or for a day after equilibrium measurements, although there was greater fluctuation in spectrophotometric readings due to settling of cells and rouleaux.

Most experiments were performed at 18° C., slightly above the upper limit of the physiological temperature range of the hagfish. However, in connection with a determination of the heat of oxygenation of this hemoglobin some studies were made at 11° C., well within the normal temperature range, and at 29–30° C.

Results

Instead of presenting all data in the form of the usual "oxygen dissociation curve," the linear transformation based on the Hill approximation,

$$y = 100 \frac{(p/p_{50})^n}{1 + (p/p_{50})^n},$$

is used in Figures 1 and 3 (Lemberg and Legge, 1949). The variables y and p are the per cent oxyhemoglobin and the partial pressure of oxygen, respectively.

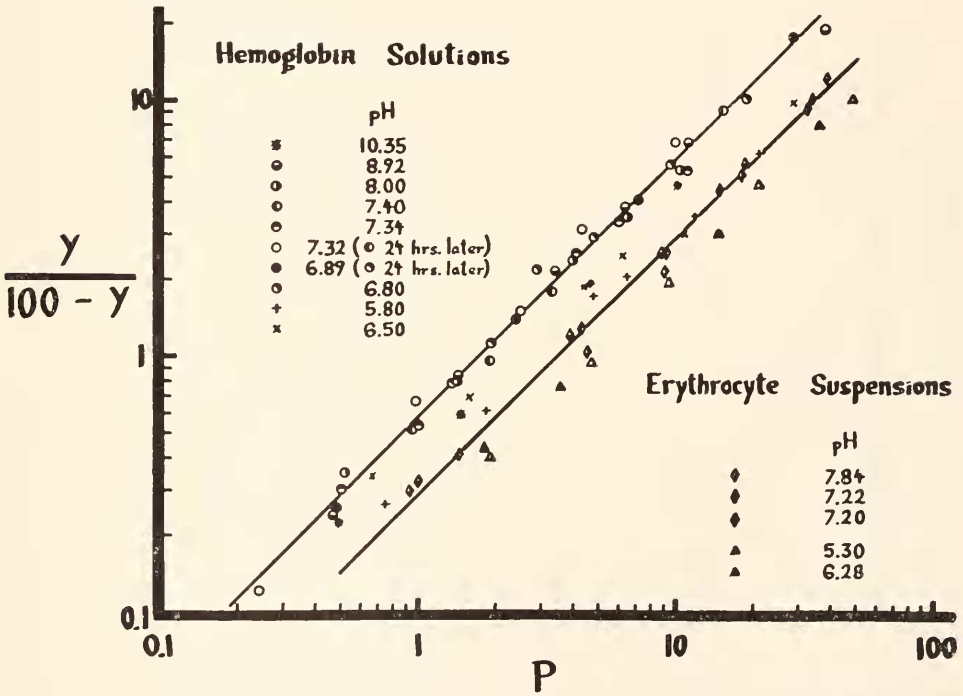

Figure 1. Oxygen equilibrium of hemoglobin of the California hagfish, *Polistotrema stouti*. Three to four per cent hemoglobin solutions in potassium phosphate buffer; final ionic strength = 0.2. Erythrocyte suspensions in isotonic phosphate-buffered saline with Karo added as explained in text. Temperature = 18° C. The solid lines are drawn arbitrarily with a slope (n) = 1.00 and a p_{50} corresponding to approximately physiological pH's.

That value of p for which y equals 50% is the p_{50}. The "sigmoid coefficient," n, is a measure of the heme-heme interactions. Hence, p_{50} is an inverse measure of the oxygen affinity, and n determines the shape of the oxygen dissociation curve. If the slope of the transformation, log $[y/(100 - y)]$ as a function of log p, is one, then the hemes are totally independent—*i.e.*, there is no heme-heme interaction. As can be seen from Figures 1 and 3, where the solid lines are drawn with a slope of 1.00, this is true of hagfish hemoglobin inside and outside the erythrocyte, and at high and low temperatures.

Between pH 6.7 and 9.0 hagfish hemoglobin in solution shows no detectable Bohr effect. Outside that pH range a significant decrease in oxygen affinity occurs; however, this effect appears to be a prelude to more drastic changes (methemoglobin formation and decrease in solubility), which become apparent several hours after equilibrium measurements. This is in contrast to the solutions at intermediate pH which are stable for days and display identical oxygen equilibria when re-analyzed one or two days after the original measurements (see Figure 1). No Bohr effect was observed for erythrocyte suspensions at pH's above neutrality; however, paralleling the behavior of hemoglobin in solution, a slight oxygen affinity decrease occurs at acid pH's. The effect was shown not only

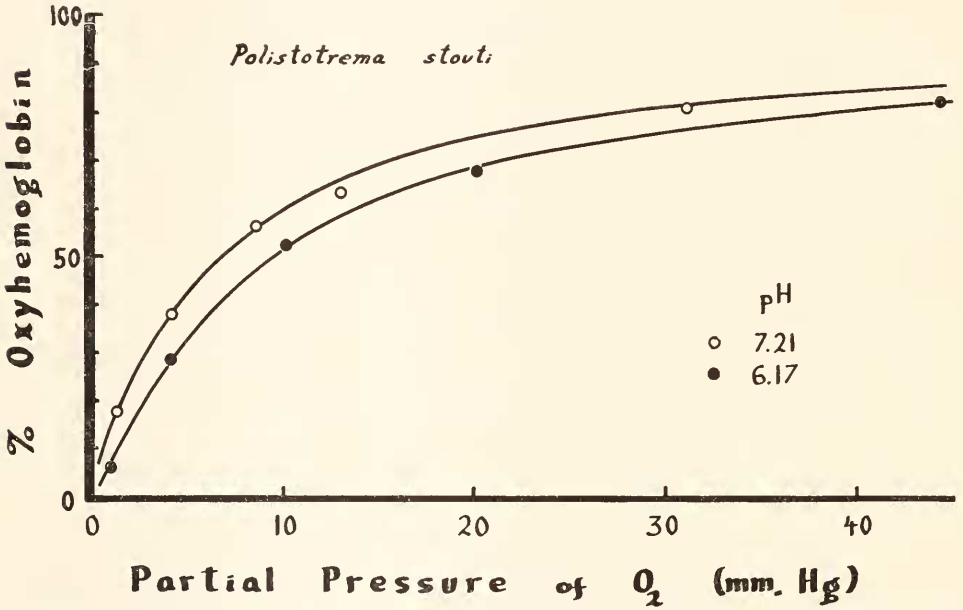

FIGURE 2. Oxygen dissociation curves of erythrocyte suspensions of the California hagfish, *Polistotrema stouti*, at two different pH's, showing the possible very slight Bohr effect. Erythrocytes in phosphate-buffered saline; no Karo present. Temperature = 20–21° C.

by the partially clarified suspensions (Fig. 1), but also when no Karo was present (Fig. 2). In contrast to hemoglobin solutions such acidic erythrocyte suspensions were stable, possibly because of the presence of cellular reducing systems able to reduce any methemoglobin. The observed decrease in oxygen affinity could represent a very small Bohr effect; however, until it is shown that the decrease in oxygen affinity is *rapid* and *entirely reversible*, the possibility of slight denaturative changes in the protein cannot be overlooked, especially in view of the results obtained for hemoglobin solutions.

The presence of CO_2 specifically decreases the oxygen affinity, in addition to its effect resulting from the increase in acidity, for hemoglobin of the horse (Margaria and Milla, 1955) and the teleost *Sebastodes ruberrimus* (Manwell, unpub-

lished data). That CO_2 does not cause any special Bohr effect for hagfish hemoglobin is shown in Figure 3.

Because the possible Bohr effect of hagfish hemoglobin is so small and occurs at almost one pH unit below the normal pH of hagfish blood (7.5–7.7; Prosser *et al*, 1950; David Jensen, personal communication), it is reasonable to assume that it is of no physiological significance, especially as CO_2 does not have any specific effect.

Knowledge of the heat of oxygenation ($\Delta H°$) of hagfish hemoglobin enables one to predict the position of the oxygen equilibrium at any particular physiological

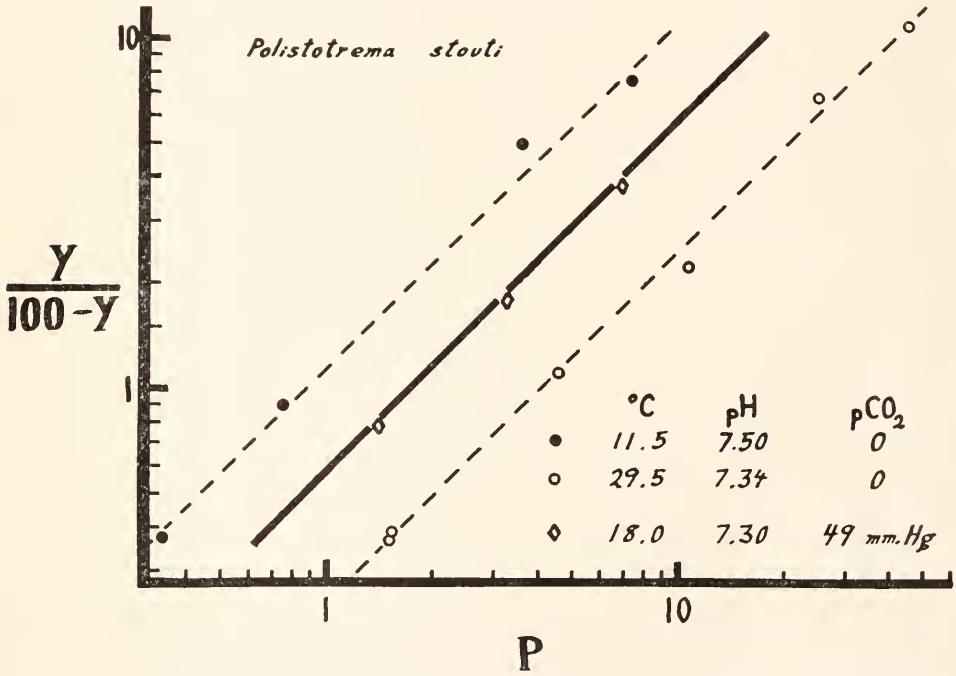

FIGURE 3. Oxygen equilibria under a variety of conditions of hemoglobin solutions prepared from the blood of the California hagfish, *Polistotrema stouti*. Dashed lines are drawn to approximate the oxygen equilibrium at 11.5 and 29.5° C. The solid line is drawn on the basis of data presented in Figure 1 in order to facilitate the comparison of hemoglobin solutions in the presence and in the virtual absence of CO_2.

temperature. In addition, absence of a Bohr effect facilitates the evaluation of $\Delta H°$, for corrections representing the effects of ionizations of heme-linked groups do not need to be applied. Using data presented in Figure 3, a value of $\Delta H° = -9.3$ kcal. (one atmosphere of dissolved oxygen gas at the standard state) was obtained as in a previous study (Manwell, 1958a). This value of $\Delta H°$ is similar to those observed for hemoglobin of sheep (-8.2 kcal.; Paul and Roughton, 1951), the holothurian *Cucumaria miniata* (-8.4 kcal.; Manwell, 1958f), and adult and fetal spiny dogfish, *Squalus sucklcyi* (-8.7 to -9.5 kcal.; Manwell, 1958d). This group of values for the heat of oxygenation of various hemoglobins is not

characterized by the extensive variation seen in older data (reviewed by Paul and Roughton, 1951); theoretical considerations imply that there be relatively little variation in these values for a particular respiratory pigment, although significant differences occur between hemoglobin, hemocyanin, and hemerythrin (Klotz and Klotz, 1955; see, also, Manwell, 1958a).

Discussion

Biochemical and Physiological

Hagfish hemoglobin has a hyperbolic oxygen dissociation curve. Beyond that point, however, resemblance to sea lamprey hemoglobin (Wald and Riggs, 1951) ceases. Hagfish hemoglobin lacks a Bohr effect over a pH range well in excess of pH's to be expected in living hagfish. The hagfish is, accordingly, the first adult vertebrate whose blood is known to lack a Bohr effect. In addition, the oxygen affinity of hagfish blood is very high—at physiological conditions as high, if not higher than that of any known vertebrate blood; the p_{50} is 2–4 mm. Hg [2] over the temperature range of 5–15° C. By way of comparison, p_{50} for human blood is 26 mm. Hg at pH = 7.3–7.4 and a temperature of 37° C. (Prosser *et al.*, 1950). *Arenicola* hemoglobin has an especially high oxygen affinity, p_{50} = 2–2.5 mm. Hg at 20° C. (Allen and Wyman, 1952); yet, by virtue of its extremely sigmoid oxygen dissociation curve ($n = 6$) *Arenicola* hemoglobin appears to be more suitable for oxygen transport than hagfish hemoglobin.

The comparison between hemoglobin solutions and erythrocyte suspensions prepared from the blood of the hagfish indicates that no specific interaction occurs between the hemes of adjacent hemoglobin molecules inside the cell. Hence, these data are consistent with—but do not necessarily establish—the idea that the molecular weight of hagfish hemoglobin *in situ* corresponds to but one heme per molecule—*i.e.*, approximately 17,000–18,000.

Comparison of the results with the introduction to this study shows that hagfish hemoglobin possesses all three of the features of the oxygenation reaction considered to be primitive by Wald (1952). However, the properties of hagfish hemoglobin, considered to be characteristic of *storage* hemoglobin by Wald, are displayed by a vascular hemoglobin, which one might accordingly assume to be involved in oxygen *transport*.

Living hagfish have been examined in an attempt to see whether there may be a significant difference in the color of blood entering and leaving the tissues and the gills, a condition which would indicate participation of the hemoglobin in oxygen transport. Unanesthetized hagfish were pinned at the extreme caudal and cranial ends (but not unnaturally stretched out) to a board immersed in oxygenated sea water at 10–12° C. The animals struggled violently until both ends were pinned down; they then remained quiescent for the duration of the experiment. A median ventral incision was made in the vicinity of the liver and the heart, care being taken to avoid cutting any blood vessels. The slime secretions were periodically removed. When slime production ceased, blood in the dorsal aorta (leaving the gills) and in various veins (leaving the tissues) was compared

[2] This approximate range of p_{50} for hagfish blood at physiological temperatures has been calculated (Manwell, 1958a) on the basis of the p_{50} for erythrocyte suspensions at 18° C. (see Fig. 1) and the heat of oxygenation ($\Delta H°$) of hagfish hemoglobin in solution.

visually with "reduced" and oxygenated standards in hagfish blood vessels. Blood in the veins appeared to be almost de-oxygenated; blood in the arteries was approximately 50% oxygenated. This condition did not change over several hours of continuous observation. By reference to the oxygen dissociation curves, it can be seen that the internal oxygen tensions were extremely low, although the hemoglobin was functional in oxygen transport. An improved physiological experimental approach would be highly desirable; however, the hagfish—considering its small size, its surprisingly violent activity when handled, and its copious slime-producing abilities—is not an especially suitable form in which to determine arterial-venous oxygen concentrations.

Wald (1952) comments (p. 367): "The business of a circulatory hemoglobin, having combined with oxygen at the body surface, is to release it in the tissues *at high tensions*. . . ." (Italics are those of Wald.) Clearly, hagfish hemoglobin is biochemically unable to function in such a way; and, the observations made on living specimens tend to strengthen the idea of oxygen transport at *low* internal oxygen tensions in *Polistotrema stouti*. Redmond (1955) has found extensive evidence for oxygen transport at low internal oxygen tensions in several decapod crustaceans. Several studies indicate that such a condition also exists in some but not all annelids (reviewed by Eliassen, 1953; see also, Jones, 1954; Eliassen, 1955; Manwell, 1958e). Adult spiny dogfish, *Squalus sucklcyi*, have a hemoglobin with a hyperbolic oxygen dissociation curve inside and outside the erythrocyte (Manwell, 1958d); yet, polarographically determined oxygen tensions of blood leaving the heart were never above 5 mm. Hg in 15 resting dogfish. Very low venous oxygen tensions have been observed in some teleosts—but not the mackerel (Black, 1951). Especially interesting in this regard is the marked *suppression* of heme-heme interaction by the erythrocytes of some teleosts and a species of holocephalian (Manwell, unpublished data); although n for clingfish *Gobiesox* hemoglobin in solution is 2.5–2.6 and thus approaches values of n for mammalian hemoglobins (2.6–3.0), inside the red blood cell the oxygen equilibrium of *Gobiesox* hemoglobin is almost devoid of heme-heme interaction ($n = 1.2$–1.4); this trend is exactly the opposite of what would be expected were the sigmoid oxygen dissociation curve always so vital for oxygen transport.

Under conditions where the tissues tolerate—or require—low oxygen tensions the properties usually associated with a transport hemoglobin would be of little selective advantage. In addition, if a large diffusion gradient were necessary to account for movement of sufficient oxygen across the epithelium of the gills or skin, then such properties as low oxygen affinity and large Bohr effect would prevent loading of the respiratory pigment with sufficient oxygen in the organ of external respiration. ("Sufficient" does not imply *complete* saturation; see Redmond, 1955.) Partial use of anaerobic metabolism could free tissues from dependence on large internal oxygen tension gradients. At the same time as such a rigorous dependence on oxygen were reduced, however, so would the metabolic efficiency decline (aerobic metabolism yielding several times more energy per unit weight of substrate than anaerobic metabolism). Consequently, one might expect large, very active animals (*e.g.*, cephalopods, some fishes, birds, and mammals) to have evolved increasing dependence on the more efficient aerobic metabolic pathways—and at the same time oxygen transport at high internal oxygen tensions. In such cases the sigmoid oxygen dissociation curve, the low oxygen affinity, and

the large Bohr effect would be of the greatest selective advantage in increasing the efficiency of the respiratory pigment. It is well known that squid hemocyanin, mammalian and avian hemoglobins, and mackerel and trout hemoglobins possess all of these characteristics (reviewed by Florkin, 1949; Prosser *et al.*, 1950).

Phylogenetic

In terms of Wald's (1952) previously mentioned theory on the origin and evolution of hemoglobin one might be tempted to infer that the primitive hagfish has retained in a hemoglobin used in oxygen transport all three oxygenation properties to be expected of hemoglobin in an earlier stage of evolution—that represented by an oxygen storage hemoglobin. However, some or all of the properties of hagfish hemoglobin may represent specialization to a particular mode of life far different from that of known fossil Agnatha. The hagfishes are, in spite of some primitive characteristics, well-adapted, biologically successful animals. Over several types of ocean bottom in temperate seas the hagfishes are among the dominant scavengers—or parasites—feeding on dead and dying fishes; they are often present in such numbers as to restrict or prevent several types of fishing operations (Young, 1950). Certain characteristics of the hagfish, such as the rasping tongue, complete absence of scales and bone, and the habit of feeding on teleost fishes, are not properties of fossil Agnatha (Ostracoderms). These features must have evolved independently of other aspects of early vertebrate phylogeny. The differences in the properties of sea lamprey (Wald and Riggs, 1951) and hagfish hemoglobin may be correlated with the well-known ecological observation: the hagfish enters, often in large numbers, the body of its prey and thus is often exposed to low O_2 and high CO_2 tensions; the lamprey remains attached to the surface of its host, thereby having well-oxygenated water of low carbon dioxide tension available for its respiration at all times. In addition, so far as is known, the hagfish does not make any sustained active movement comparable to the anadromous migration of the sea lamprey.

Several other objections to Wald's (1952) theory in its present form can be raised:

(1) Cytochrome oxidase has been considered the phylogenetic precursor of hemoglobin because: (a) it combines reversibly with CO and reacts with O_2; and, (b) beef heart cytochrome oxidase has an extremely high oxygen affinity, no Bohr effect, and an almost hyperbolic equilibrium curve with CO—all properties that a "primitive" hemoglobin ought to possess (Wald and Allen, 1957). Unfortunately, neither the prosthetic group (Paul, 1951; Stotz, Morrison and Marinetti, 1956) nor the protein moiety (Lemberg and Legge, 1949) of this respiratory enzyme (or enzyme complex) resembles the corresponding parts of hemoglobin as closely as might be desired. Cytochrome *c* would be a better, although not entirely satisfactory, hemoglobin phylogenetic precursor. At least its prosthetic group is the same as that of hemoglobin, although linked to the protein differently; and, its protein moiety is readily water-soluble, although of lower molecular weight (one heme per 13,000–15,000) and higher isoelectric point (pI = 10) (Paleus, 1955) than any known hemoglobin. When the heme of cytochrome *c* is not completely protected by coordination of the iron with the imidazole groups of two

histidine residues, the enzyme combines with CO and is oxidized by O_2 (Lemberg and Legge, 1949; Theorell, 1956). Bartsch and Kamen (1958) isolated a bacterial heme protein—originally called a "pseudohemoglobin"—which resembles cytochrome *c* in many respects, although its isoelectric point (pI = 5) is comparable to that of invertebrate and cyclostome hemoglobins (Prosser *et al.*, 1950) and it is readily oxidized by O^2 and combines reversibly with CO. The carbon monoxide reaction of this bacterial heme protein is *not* invariant to pH change—in contrast to cytochrome oxidase (Wald and Allen, 1957). In support of *some* connection between the syntheses of cytochrome and hemoglobin is the finding of Ycas (1956) that aerobically grown yeast in the presence of antimycin produces less cytochrome *a* and more hemoglobin than controls; however, as Ycas suggests, this relation may be explained by assuming that the heme of hemoglobin is a precursor to the modified heme of cytochrome *a*. At present there is so little comparative biochemical information on the cytochromes and other heme-containing enzymes that one cannot rule out the possibility that the proteins of various hemoglobins have arisen from apoenzymes of quite unrelated biocatalysts; certainly, the protoheme prosthetic group is always phylogenetically available. Several proteins besides globin will combine with heme, although none are yet known that will enable this heme to combine reversibly with molecular oxygen (Lemberg and Legge, 1949).

(2) Wald (1952) states (p. 369): "The hemoglobins that have arisen so sporadically among invertebrates of various orders are all storage hemoglobins." However, oxygen transport by hemoglobin occurs in several annelids (Johnson, 1942; Eliassen, 1955; reviewed by Eliassen, 1953; Manwell, 1958e), the brine shrimp *Artemia* (Gilchrist, 1954), and even such small arthropods as daphnids (Hoshi, 1957). As the experiments of Redmond (1955) show, the presence of a respiratory pigment in the blood of invertebrates in low concentration does not rule out significant oxygen transport by that pigment. Coelomic hemoglobins, such as those of *Urechis* (Redfield and Florkin, 1931) and *Cucumaria miniata* (Manwell, 1958f), are usually assumed to function in oxygen storage; however, the movement of coelomic fluid, either by muscular contraction or cilia, presents the possibility of oxygen transport by the coelomic hemoglobin from cloacal diverticula (*Urechis*) or respiratory trees (*Cucumaria*) to tissues in or adjacent to the coelom.

(3) That a hyperbolic oxygen dissociation curve, high oxygen affinity, and no Bohr effect should represent primitive conditions (Wald, 1952) requires comment. The properties of the oxygen equilibrium of the vertebrate storage hemoglobin (myoglobin) rest on studies of crude extracts or purified preparations prepared from the muscles of five species of mammals (reviewed by Lemberg and Legge, 1949; see also, Rossi-Fanelli and Antonini, 1958). As Lemberg and Legge point out, the oxygen equilibrium of myoglobin *in situ* in the muscle remains to be evaluated. The findings, that *n* could be as high as 1.6 for oxygen equilibria of extractions of *Cryptochiton* myoglobin (Manwell, 1958c) and that *n* could be as high as 2.8 in the reaction of horse metmyoglobin with various ligands (Kiese and Kaeske, 1942), indicate that heme-heme interactions can exist under certain conditions in tissue hemoglobins. One would expect tissue hemoglobins to have a high oxygen affinity because of limitations on the intracellular oxygen tensions imposed by the combination of passive diffusion of oxygen and aerobic cellular

metabolism. In the case of *Cryptochiton* even when the oxygen dissociation curve of the radular myoglobin is sigmoid, it lies far to the left of the corresponding curve for the vascular hemocyanin; hence, the presence of heme-heme interactions in the myoglobin does not interfere with the functional oxygen transfer system (Manwell, 1958c). Interactions between oxygen-affine centers have evolved in all four major classes of respiratory pigments (hemoglobin, hemocyanin, chlorocruorin, and hemerythrin); Bohr effects are found in all these classes except hemerythrin (reviewed by Prosser *et al.*, 1950).[3]

There is reason to believe that heme-heme interactions and the Bohr effect are not necessarily specialized acquisitions restricted to respiratory pigments in an advanced state of evolution but are expressions of very basic properties found in many unrelated proteins. The frequently observed variation of enzyme kinetics as a function of pH often involves interaction between proton-affine centers on the protein moiety and the active center (Alberty, 1956). Heme-heme interaction, likewise, has its parallel in the interaction between centers having similar reactivities in proteins possessing two or more such sites per molecule. Such interactions occur in the binding of dyes and ions to some multivalent proteins (Klotz, 1954) and in the kinetics of some enzymes (Botts and Morales, 1953).

Finally, the ease with which certain reagents (various mercurials, formaldehyde, and glutathione) will remove the heme-heme interactions, partially restore those interactions, greatly increase the oxygen affinity, and/or modify the Bohr effect (Guthe, 1954; Riggs and Wolbach, 1956) implies that these properties are not invariant for a particular hemoglobin molecule. In addition, the differences in the oxygen equilibrium of some hemoglobins inside and outside the red blood cell (Root, Irving and Black, 1939; Manwell, unpublished data) indicate also that a considerable lability exists with regard to the properties of the oxygen equilibrium.

It seems reasonable to assume that the phylogenetic order of first appearances was: heme-containing respiratory enzymes, tissue hemoglobins, vascular hemoglobins. However, the present discussion indicates the difficulty of knowing (a) if a certain set of characteristics of the oxygen-hemoglobin equilibrium—*e.g.*, high oxygen affinity, no heme-heme interactions, and no Bohr effect—is basically primitive, and (b) if any particular component of the cytochrome system or any other heme-containing enzyme is evolutionally the forerunner of hemoglobin.

Summary

1. Oxygen equilibria of hagfish hemoglobin inside and outside the red blood cell have been obtained under a variety of conditions. The oxygen affinity of the hemoglobin in the erythrocyte suspensions is high (p_{50} = 3–4 mm. Hg at 18°), although it is even higher in hemoglobin solutions (p_{50} = 1.8 mm. Hg at 18° C.). There is no interaction between hemes (n = 1.00) and virtually no Bohr effect. The effect of temperature on the oxygen equilibrium of hagfish hemoglobin is

[3] Absence of the Bohr effect has been confirmed for various sipunculid coelomic hemerythrins (Manwell, 1958a, and unpublished studies on *Dendrostomum zostericolum* and *Siphonosoma ingens*); however, the coelomic hemerythrin of the brachiopod *Lingula*, a form that is morphologically essentially unchanged since the Cambrian period, has a Bohr effect that is two-thirds the magnitude of that observed for human adult hemoglobin (Manwell, 1958, unpublished experiments)!

similar to that observed in recent experiments on other hemoglobins ($\Delta H^\circ = -9.3$ kcal. for hagfish hemoglobin).

2. Several aspects of Wald's (1952; see, also, Wald and Allen, 1957) theories on the evolution and function of hemoglobin are criticized in view of these data on hagfish hemoglobin and on the basis of information in the literature. It is concluded that: (1) At present there is no reason to favor cytochrome oxidase as *the* phylogenetic precursor of hemoglobin. (2) Many invertebrate hemoglobins function in oxygen transport. (3) If the internal oxygen tensions are sufficiently low, a respiratory pigment participating in oxygen transport does not need to possess a low oxygen affinity, a sigmoid oxygen dissociation curve, and a marked Bohr effect. (4) It is impossible to say if a particular set of properties of the oxygen equilibrium is basically "primitive." (5) Physiological conclusions on hemoglobin should be made upon studies of the pigment *in the natural condition—i.e.*, myoglobin in the muscle, or intracellular vascular hemoglobin in the erythrocyte.

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